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# Administration of aromatase inhibitor MPV-2213ad to blue fox vixens (*Vulpes lagopus*) as a model for contraception in female dogs<sup>☆</sup>

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## ABSTRACT

The interest in non-surgical approaches to contraception and fertility control in female dogs has increased in recent years. In this study the effect of an aromatase inhibitor (finrozole) was evaluated in fur production animals, farmed blue fox vixens, as a model for contraception in bitches. A total of 80 vixens were divided into 4 groups, receiving orally placebo (A) or finrozole 0.5 mg/kg (B), 3.5 mg/kg (C) or 24.5 mg/kg (D) for 21 consecutive days beginning in the pre-ovulatory period of heat. Monitoring of the vixens included clinical signs of heat, measurement of vaginal electrical resistance (VER) as well as oestradiol and progesterone concentrations in plasma. The approximate relation of the start of treatment to ovulation varied from 11 days before to one day after ovulation provided that the LH peak occurred 0.5–2 days before the VER peak and ovulation was then estimated to occur 2 days after the LH peak. Seventy vixens were artificially inseminated within 8 h after a 50  $\Omega$  decline in vaginal electrical resistance was detected. Ten vixens were not inseminated. Pregnancy was confirmed by transabdominal ultrasound examination and birth of cubs was recorded. The pregnancy rates in the groups were 89.5% (A), 81.3% (B), 55.6% (C) and 52.9% (D). The average number of live born pups in the four groups was 9.4 (A), 7.0 (B), 5.8 (C), and 3.8 (D), respectively. No deleterious effects (for instance malformations) of finrozole on pups could be verified. The administration of finrozole did not have a significant effect on oestradiol parameters and VER values in vixens. Progesterone values were significantly higher in treatment groups compared with the placebo group. The results indicate that pregnancy could be avoided by finrozole provided that doses of  $\geq 3.5$  mg/kg were used and the treatment was initiated at least four days before the day of artificial insemination. This corresponds with two to six days before ovulation provided that the LH peak occurred 0.5–2 days before the VER peak and that ovulation then occurred in average 2 days after the LH peak.

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## 1. Introduction

Due to a steady increase in dog populations worldwide, there is an increased awareness amongst dog breeders and dog owners towards controlling reproduction. The most common way of controlling reproduction in dogs is surgical removal of the gonads. This, however, is not an option for dog breeders who wish to use their dogs for future breeding. In bitches, possibilities for contraception, defined as suppression of oestrus, are based on hormonal treatments. Hormonal contraception in bitches has included the use of

<sup>☆</sup> Part of the results of this study have been presented as abstracts and posters in three international conferences: the 12<sup>th</sup> International Congress of the European Association for Veterinary Pharmacology and Toxicology (EAVPT 2012), 20<sup>th</sup> Annual ESDAR and 13<sup>th</sup> AERA Conference 2016 (ESDAR-AERA 2016) and the 1<sup>st</sup> European College of Animal Reproduction (ECAR) conference 2019.

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progestins, androgens or gonadotropin releasing hormone (GnRH) analogs that act either directly by blocking reproductive hormone receptor-mediated events or indirectly block conception via negative feedback mechanisms [1]. However, many of these treatments are associated with a high incidence of side effects, which can be detrimental for individuals intended for breeding in the future. Other possibilities of contraception in bitches are via activation of the immunological system with vaccinations against GnRH, the luteinizing hormone (LH) receptor and the zona pellucida proteins [2].

Compounds for prevention or modification of oestrogens are classified as anti-oestrogens. Some of these are GnRH analogs and aromatase inhibitors that block oestrogen synthesis [3]. Another anti-oestrogenic group of drugs are oestrogen receptor blockers [4]. Knowledge of the effect of receptor blocker anti-oestrogens in dogs is still scarce. Most studies have been carried out using tamoxifen, which seems to act more like an agonist than antagonist, inducing signs of heat in bitches [3].

Another possibility for contraception in female dogs might be the use of finrozole, a non-steroidal aromatase inhibitor. The effect of finrozole has been evaluated for treatment of urinary disease in men [5]. Due to a high demand of reversible contraception in veterinary medicine for female dogs, several yet unpublished studies were initiated by the sponsor in order to investigate the function and mechanism of finrozole. The idea was to develop a compound for prevention of heat and for contraception in female dogs, without side-effects related to the use of, for instance, gestagens.

Aromatase is a cytochrome p450 enzyme which catalyses the conversion of androgens to oestrogens [5]. Aromatase activity is increased when oestradiol-17 $\beta$  concentration is elevated in response to FSH inducing follicular growth. Aromatization is the rate limiting step in oestrogen formation and a target for development of agents to block the synthesis of oestradiol [6]. Blockade of this step allows treatment of diseases dependent of oestrogens, such as for instance hormone dependent breast cancer in human medicine [7]. Use of aromatase inhibitors has also been studied in treatment of endometriosis related pain in humans [8]. In veterinary medicine, aromatase inhibitors have been used for treating diseases of the prostate gland in male dogs, since oestrogen plays an important role in the development of benign prostatic hyperplasia (BPH) [9].

In a study by Yapura et al. [10] the potential of non-steroidal aromatase inhibitors as an alternative for controlling ovarian function in cattle has been investigated. Letrozole treatment was found to extend the lifespan of the dominant follicle resulting in a delay in the emergence of the next follicular wave or in delayed ovulation [11]. Letrozole treatment in cattle had a consistent luteotrophic effect and treatment was associated with development of a larger CL and/or higher circulating concentrations of progesterone [12].

Maintaining dog populations for research purposes raises several ethical issues, as one of the strongest objections is the use of a pet animal for research. To avoid this, a closely related canine species was selected as a model, under clinical field trial conditions, without sacrifice of the animals. Farmed blue fox vixens (*Vulpes lagopus*) are fur production animals, and housed in individual pens on farm conditions since their weaning (age about 8 wks). As they present hormonal patterns similar to those of female dogs [13] they are suitable as model species for domestic dogs.

Farmed blue foxes, colour mutations of the arctic fox, are monoestrous seasonal breeders, with reproductive activity during the spring months of February to May. The oestrous cycle of a blue fox vixen is divided into four stages; pro-oestrus, oestrus, metoestrus and anoestrus. As described by Farstad [14], no physical signs of approaching heat can be detected, on average, until two

weeks before the LH-peak, indicating the start of pro-oestrus, the period of follicle growth in ovaries. Before this, approximately one month prior to the LH peak, the first follicles appear on the ovarian surfaces [15]. Clinical signs of early follicular development are swelling of the vulva, increased frequency of urination and vocalization. The duration of pro-oestrus is usually one week. However, it may extend to 12–24 days and may be shorter in mid-to late season than early in the season [14].

The majority of the vixens come into oestrus, i.e. are willing to be mated, in mid-March to mid-April [14]. The duration of oestrus ranges between one to 8 days, the average being 4–5 days [16]. The maximum swelling of the vulva is usually observed on the day of onset of oestrus [14]. The onset of oestrus in vixens may vary considerably in regard to the LH peak and ovulation and may start simultaneously with the LH peak, a day after the LH peak or even at ovulation two days after the LH peak [17,18]. Oestrus is characterized by ovaries containing late preovulatory follicles or ovulated follicles and by ovarian steroid production shifted from predominantly oestrogens to progesterone [14]. Ovulation, occurring within the period of oestrus, is followed by a period of extended luteal function. Metoestrus is defined as the luteal phase in the blue fox. In pregnant and non-pregnant vixens, the whole period from ovulation to regression of corpora lutea is termed metoestrus. After metoestrus, anoestrus begins, lasting on average for up to 9 months and indicating the period of ovarian rest [14].

Peak plasma oestradiol values in vixens coincide with the LH peak, i.e. 1–2 days before the onset of oestrus. Electrical resistance of the vagina varies during oestrus [19]. In most vixens, the electrical resistance reaches the peak 0.5–2 days after the LH peak [17,18], close to the first day of sexual receptivity. The decline phase of the resistance occurs 2–6 days after the LH peak. Ovulations occur spontaneously 1–3 days after the LH peak (on average: LH + 2 days) and oocytes are ovulated in the developmental stage of primary oocytes [17,20]. The length of gestation in blue foxes is approximately 52 days from mating to parturition but may vary between 48 and 57 days, depending on whether the initiation of the gestation is calculated from the time of the LH peak, ovulation or fertilization [14].

The aim of the present study was to evaluate the contraceptive effect of oral administration of finrozole in vixens as a model of contraception in bitches. Determination of an efficacious dose of finrozole for prevention of pregnancy was performed by assessing the concentrations of oestradiol and progesterone and the incidence of pregnancies.

## 2. Materials and methods

### 2.1. Animals

The study was a placebo controlled open study, with 80 vixens (*Vulpes lagopus*) selected out of 190 individuals during the breeding season of 2009. Selection of 1.5–4.5 year old vixens, that had one to four previous whelpings, was done based on body condition (with score 3 by Kempe et al. [21]), and weight ranging from 6.7 to 10.9 kg. Large sized (>11 kg) vixens were excluded as they are more likely known to be barren or to lose their pups during pregnancy or right after birth [22]. Because all animals had been raised together since birth, the acclimatization period could be reduced and began 14 days before the anticipated beginning of the heat. For inclusion into the study, the vixens were first examined in the end of February and twice in March by the responsible study veterinarian in order to exclude animals with pathological conditions that could interfere with the outcome of the study. Every vixen was individually weighed and marked with a microchip (AVID Friendship™ by Avid Identification Systems, Inc. California, USA), enabling

identification at all times with AVID MiniTrackKer II reader (Avid Identification Systems, Inc. California, USA). The vixens were dewormed with ivermectin (Ivomec Comp, Merial SAS, France, 1.5 kg Ivomec/1000 kg feed) twice at 4 day interval before the beginning of the study. All procedures were conducted under approval from the Animal Experimental Board in Finland (ELLA, decision STH904A, licence 8.12.2008ESLH-2008-09367/Ym-23).

### 2.1.1. Housing and general management

All vixens were housed individually in an outdoor shed at MTT Agrifood Research Finland facility in Kannus, Finland, in cages (Length 110 cm x Width 105 cm x Height 70 cm). Each cage included a wire-mesh shelf (Length 105 cm x Width 25 cm), enabling the vixens to climb and exhibit species like behaviour. For this purpose, the vixens were also provided with an activity object, such as a piece of wood, to play with during the entire duration of the study. For whelping, the vixens were provided with a wooden whelping nest box in their cages.

The vixens were fed on a restricted breeder's diet in order to optimise breeding results [23]. The vixens were fed once daily, January to March at 2 pm–4 pm and April to June at 10 am to 5 p.m. The amount of feed distributed daily ranged between 150 and 600 g. Fresh water was provided by the municipal water supply via a non-freezing automatic water supplier.

### 2.1.2. Outdoor temperature and hygrometry

The variation in outdoor temperature was between  $-24^{\circ}\text{C}$  and  $+28^{\circ}\text{C}$  and the hygrometry 20–100% depending on the weather and the time of year. A day/night cycle of 11 h of light and 13 h of dark per day was maintained in March and early April. During summer the hours of daylight increased from 12.5 h in March to 20 h and 20 min in June.

## 2.2. Allocation of groups

A total of 80 vixens were randomly allocated into four treatment groups (20 vixens per group) according to the oral administration of either placebo or finrozole in different doses: group A (placebo), group B (0.5 mg/kg), group C (3.5 mg/kg) and group D (24.5 mg/kg).

The progression of heat was monitored by measurement of vaginal electrical resistance (VER) in order to define the start of treatment. The original purpose was to begin administration of the aromatase inhibitor when an ascent in VER values was detected indicating ovarian follicular activity [14,18]. Due to an earlier onset in the breeding season of many individuals from the original research population of 190 vixens, this selection criterion was changed to include the vixen into the study so that she had received at least one dose of treatment before she was artificially inseminated. This meant the vixen had eaten at least one dose of treatment in the morning if she was inseminated in the afternoon. In our data, we had two vixens whose artificial insemination (AI) and initiation of treatment occurred on the same day (Fig. 1).

From each treatment group of 20 vixens animals were randomly picked into four subgroups of 20 vixens (five from each group A, B, C and D) according to the time points of administration initiation of finrozole. In the first, second, third and fourth subgroup, administration of finrozole was initiated on March 26<sup>th</sup> 2009, March 27<sup>th</sup> 2009, April 2<sup>nd</sup> 2009 and April 3<sup>rd</sup> 2009, respectively.

The preliminary study, during which this data was collected, was designed as a pharmacological dose determination study. For this specific publication, however, in order to evaluate parameters of reproductive physiology, the data was re-organised and analysed taking into consideration the stage of heat in relation to the initiation of treatment. By this, the four groups A, B, C and D were further re-organised into Placebo (A, placebo pregnant), and

treatment groups (BCD, pregnant and BCD, not pregnant). The placebo, not pregnant group, consisted of only two vixens, whose VER measurements and hormone concentrations are not shown in the graphs. The pregnancy rates in each treatment group were assorted on the basis of the number of days between initiation of treatment and first AI as the length of this period affected the pregnancy results.

## 2.3. Monitoring of heat related ovarian activity

The onset of ovarian follicular activity was detected by clinical observation of swelling of external genitalia. Controls of vixens regarding the initiation of vulval swelling was performed twice weekly beginning in March. After detecting the first sign of vulval swelling, the further process was documented daily by rating the swelling degree with a 0 to 3 scale, where 1 stands for the first clinical signs of increasing oestrogen secretion, 2 stand for medium swelling and 3 for maximum swelling indicating maximum oestrogen secretion with a VER value  $\geq 200 \Omega$ . Measurement of the electrical conductivity was performed using a modified Ohm-meter (SiLi Heat-detector, Lima A/S, Sandnes, Norway). It was started when scale 3 vulval swelling had been reached in order to monitor the rise in VER indicating the changes in vaginal secretion due to decreasing oestrogen concentrations. Measurements were continued daily until the day of first AI, indicated by a decrease of at least  $50 \Omega$  in VER values.

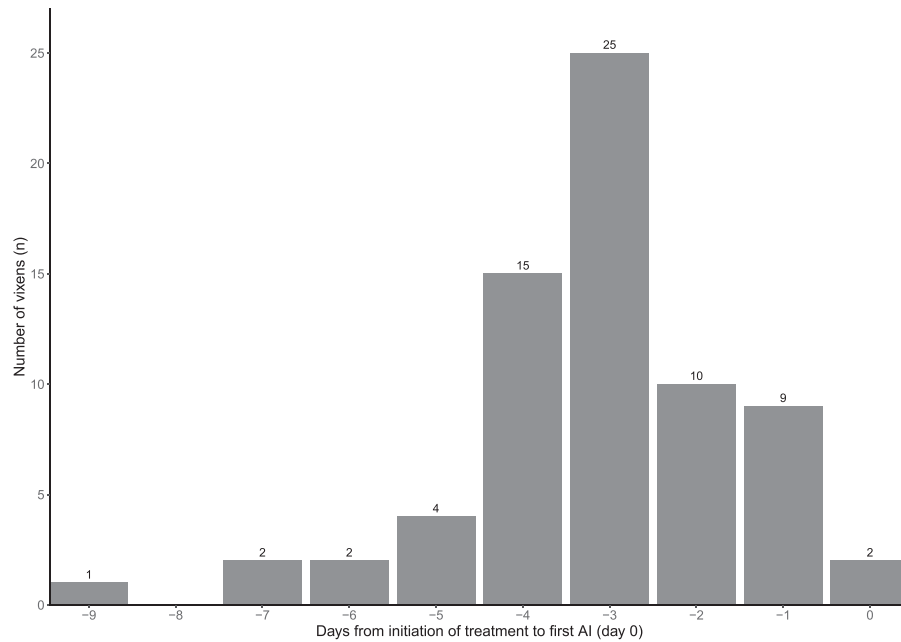
## 2.4. Administration of finrozole

The aromatase inhibitor was administered orally once a day for 21 consecutive days. Finrozole was available in capsules packed in labelled plastic jars, stored at  $15\text{--}25^{\circ}\text{C}$ . Each vixen received one capsule once a day. The capsules were opened and the content was mixed in a small amount of feed. The feed containing the medication was fed to the vixens prior to their daily ration. All vixens were monitored during feeding to ensure sufficient oral administration. Incomplete consumption of the feed was observed in 7 vixens (2 in group B, 1 in group C, and 4 in group D). One vixen per group B and C and two vixens in group D refused feeding once. Two vixens in each of groups B and D refused feeding on three different occasions. One vixen in group D refused consumption of the feed during 5 days. The timeline of incomplete administration of the compound ranged sporadically from day 8 to day 21 of administration. Because only one vixen whose treatment was initiated 1 day before first AI gave birth (of the 7 vixens associated with incomplete consumption of the feed) and because the refusals occurred during the last two weeks of administration of finrozole, those individuals were considered not to have affected the results. Necessary precautions were taken during drug administration by wearing single-use disposable clothing during handling of capsules, mixing the content into the feed and administration of the feed.

## 2.5. Semen collection and AI

Males of proven fertility were used as semen donors. Semen was collected every other day throughout the breeding season. The males and the vixens were transported from their cages to the semen collection and insemination facility using individual cages for each animal. Maximum of three males were collected at a time and the freshly collected non pooled semen was used to inseminate the vixens. If more semen was required for the day's inseminations, then three new males were picked up for semen collection.

An unsedated male at a time was placed in a semen collection stand and the second fraction of the ejaculate (0.2–1 ml of undiluted semen) was collected by digital manipulation into a glass vial



**Fig. 1.** Distribution of vixens (n = 70) regarding the initiation of treatment before first AI.

which was stored in a +30 °C incubator prior to semen collection. According to the colour and consistency of the collected semen, the fraction was diluted with EDTA (stored at room temperature in +20 °C) up to a total volume of 2–3 ml. Watery fractions were discarded, thick milky fractions were diluted with up to 3 ml of EDTA and less milky fractions with up to 2 ml of EDTA. A small sample of diluted semen was analysed under a light microscope (x 400) to check the motility of the spermatozoa. Sperm concentration was not counted. For AI, diluted semen with at least 70% progressively motile sperm was required. Prior to AI, the diluted semen was stored at +20 °C from 30 min to 3 h between inseminations.

Transcervical insemination was performed using a Norwegian catheter [24,25]. Sterile catheters and sheaths covering the metal catheters during vaginal introduction were stored at +30 °C prior to AI. An unsedated vixen was fixed by the neck and tail in an insemination rack. The vulva was disinfected with 70% EtOH before inserting a sheath into the vagina, through which the Norwegian catheter was guided to the opening of the cervix. After passing the cervix, 1 ml of fresh diluted semen was deposited into the uterus with a syringe.

According to the normal routine on fox farms, the vixens are predominantly inseminated once or twice. Based on the present data and current literature [25], it seems that one insemination is enough if the vixen has a clear rise and fall VER curve. If VER remains high for a longer time, a second insemination ensures that timing will be better with respect to oocyte maturation. The second insemination occurs two days after the first insemination according to the VER values. This routine was chosen in the present study although it meant different numbers of AIs per vixen. Finally, 52 vixens were inseminated once, 17 vixens were inseminated twice (4 in group A, 5 in group B, 3 in group C and 5 in group D), and one vixen (group A) was inseminated four times due to a two-phase resistance curve. Ten vixens were not inseminated. In eight of them (4 in group B, 1 in group C and 3 in group D), the intravaginal insertion of the probe of the heat detector was impossible due to vulval swelling progressing only to scales 1 and 2. The remaining two vixens were not VER measured and were not inseminated due to a human error (group A) and due to an injured vagina (group C).

## 2.6. Blood sampling

In each vixen two weekly blood collections were performed throughout the first three weeks of administration. Thereafter sampling was reduced to a once a week schedule, resulting in a total of 11 blood samples per vixen. All vixens were anaesthetized during collection of blood samples. For blood sample collection the vixens were transported to the blood collection facility in a transport cage system containing individual cages for each vixen. The vixens were anaesthetized with a combination of medetomidine (Dorbene 1 mg/ml, Laboratorios SYVA S.A., Spain) and tiletamine-zolazepam (Zoletil 100, 250 mg/ml + 250 mg/ml, Virbac, France). Five ml of medetomidine were mixed with tiletamine-zolazepam powder. Each vixen was treated with 0.2 ml of this combination intramuscularly containing 0.2 mg of medetomidine and 20 mg of tiletamine-zolazepam. Anaesthesia lasted for 30 min. Blood was collected from *vena saphena* after clipping a small area of fur and wiping the skin with 70% EtOH.

Four ml of blood was collected into two lithium heparin tubes (Terumo Europe N.V., Leuven, Belgium) which were centrifuged 3000 rpm for 10 min and plasma transferred into two separate tubes, each containing at least 0.5 mL of plasma. For shipment to the diagnostic laboratory at the National Veterinary School in Lyon, France for hormone analyses, the samples were placed on dry ice.

### 2.6.1. Oestradiol

For oestradiol, the samples were assayed by using a Packard RIA STAR solid scintillation counter. Oestradiol assays were performed using the kit ESTRADIOL-2 (Clinical Assays) supplied by Diasorin, 92160 - Antony (France), validated for dogs, without any changes to the usual kit protocol. The analytical sensitivity, calculated as the apparent concentration of analyte which was distinguishable from the zero calibrator (two standard deviations below zero) was 5.0 pmol/L, the intra-assay coefficient of variation ranged between 2.6% and 6.1%, the inter-assay coefficient of variation ranged between 4.6% and 6.1%. Samples for which the apparent concentration was below the analytical sensitivity of the assay were assigned to a concentration equal to zero. All the analyses were performed with



the same batch of reagents (ref 122 876) and controlled with the same control serum (CE, target values: from 336 to 504 pmol/L).

### 2.6.2. Progesterone

Progesterone was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay, using the reagents Immulite 2000 Cortisol L2KPW2 provided by Siemens Healthcare Diagnostics SAS and validated for dogs. The analytical sensitivity (quantification limit) for the assay was 0.7 nmol/L and the working range is from 0.7 to 127 pg/mL. The within-run and total precisions range between 9.5% (21.3 nmol/L) and 21.7% (0.46 nmol/L). Samples for which the apparent concentration was below the quantification limit of the assay were assigned a concentration equal to zero. All the analyses were performed with the same batch of reagents (ref 304) and controlled with the specific steroid controls of Siemens (CON 6, batch 022, three levels).

### 2.7. Confirmation of pregnancy

During the 8th, 9th or 10th blood sampling occasion corresponding to the period D23 to D46 after first AI, the anaesthetized vixens (54 out of the 70 inseminated vixens. In the remaining 16 vixens, whelping was the verification of pregnancy) were sonographically examined for pregnancy by an experienced veterinarian. Each vixen was placed on an examination table in dorsal recumbency and the abdominal fur was sprayed with alcohol. Then a 7.5 MHz linear probe of an Aloka SSD-DX Scanner (Tokyo, Japan) was inserted on the abdominal skin and the uterus was scanned for detection of foetuses. If live foetuses or clear signs of foetal resorption were detected, conception was considered to have occurred. All vixens were monitored for the delivery of pups starting the day after the expected day of parturition (D52 after first AI). The number of pups was recorded at birth and again one week later. At birth, pups were inspected by lifting the roof of the wooden whelping nest box. Dead pups were removed and examined for malformations. At one week of age, during gender determination, each pup was lifted from the nest for examination of malformations. Pregnancy rates were calculated based on both ultrasonography confirming pregnancy and pups born.

### 2.8. Statistical analysis

All data were first subjected to testing for normal distribution. Transformation (log 10) of data was used where appropriate. Oestradiol and progesterone profiles and electrical resistance in vaginal mucus were analysed using analysis of variance with repeated measurements (split-plot ANOVA, [26]; software: IBM SPSS Statistics version 24, release 24.0.0.0). In the General Linear Model for repeated measures, treatments ( $n = 4$ ) were considered as between subject factors (fixed effect) and periods ( $n = 5$ ) as within subject, repeated factors (days, every two days together forming a period). As a post testing procedure for oestradiol and progesterone concentrations at given points in time, the Tukey procedure was used for pairwise comparisons and the Dunnett procedure for timewise comparisons. Statistical testing for pup losses between placebo and treatment groups was done using ChiSquare.

The effect of treatments ( $n = 4$ ) on pregnancy outcome was analysed through ChiSquare testing. The effect of the treatments on litter size was tested through analysis of variance where treatments were used as independent factor and number of pups as outcome variable. Thereafter, multiple comparisons were carried out by Student's t-test where appropriate.

## 3. Results

The numbers of inseminated, pregnant and whelping vixens recorded in each group are presented in Table 1. The 10 vixens confirmed to be pregnant at ultrasound examination but with no verified parturition have been included in the group of pregnant vixens regarding VER and hormone concentrations shown in Figs. 2–4.

On the day of initiation of the treatment, in the majority ( $n = 48$ ) of the 70 inseminated vixens, VER measurement had been performed for at least 2 days. In eight vixens, VER had been determined just once, while 14 vixens were still at the stage of vulval swelling (11 on scale 3, three on scale 2) and thus VER measurement had not started. The mean period of VER measurements between initiation of treatment and the first AI was  $3.7 \pm 1.2$  days. In the placebo group (A,  $n = 19$ ), this period was  $4.3 \pm 1.1$  days, almost one day longer than in vixens of groups B,C and D ( $n = 51$ ) ( $3.5 \pm 1.2$  days).

As shown in Fig. 2, administration of the aromatase inhibitor finrozole did not affect the pattern of VER before AI in either treatment group when compared with group A ( $P > 0.20$ ).

During the 21 day treatment period no significant differences were found in the plasma oestradiol concentrations between the placebo group and the treatment groups ( $p = 0.70$ ) (Fig. 3). The levels of plasma oestradiol concentrations appeared normal in all groups, following a typical pattern of vixens during heat. In the pregnant and not pregnant treatment groups (BCD) and in the placebo group (A), mean SD values before first AI and after first AI were 21.3 (BCD pregnant), 33.4 (BCD not pregnant), 22.7 (placebo) and 8.8 (BCD pregnant), 21.2 (BCD not pregnant), 11.7 (placebo), respectively.

Mean progesterone patterns in the treatment groups resemble that for placebo treated control vixens (group A). However, mean progesterone values were significantly higher ( $P = 0.023$ ) both in the pregnant and not pregnant treatment groups (BCD) compared with the placebo group (Fig. 4).

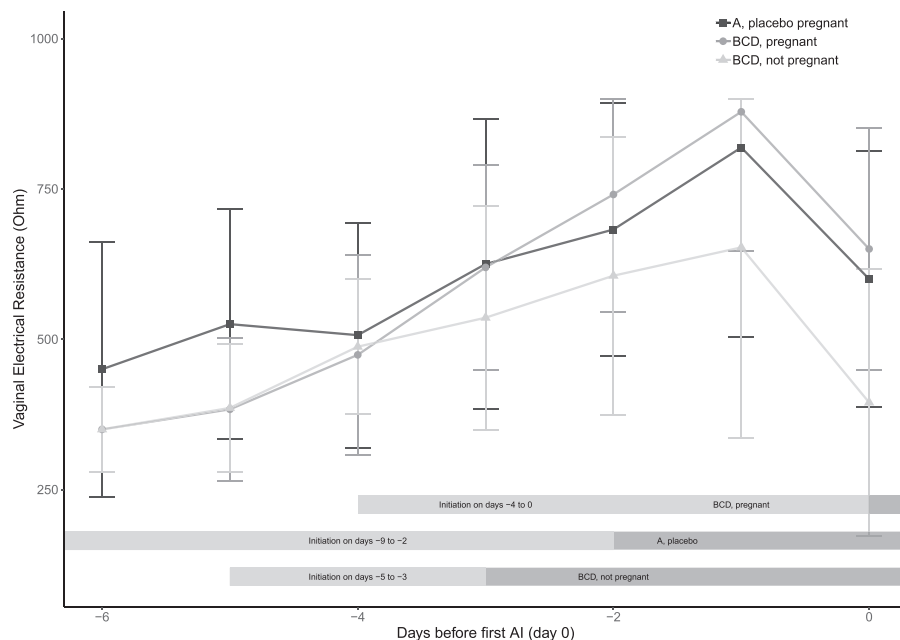
The time difference between the peaks of oestradiol is shown in Fig. 5. In this graph, values originating from blood samples and VER measurements collected before the start of treatment are included. The oestradiol concentration peaked five days before first AI in the pregnant vixens of both the placebo group A and the pregnant group BCD. In the not pregnant vixens of the treatment groups BCD, the values were still rising on day -4 and no peak was yet evident. In the BCD pregnant group, the time difference between the oestradiol and VER peaks was four days. Mean VER values of days -2 to 0 of the pregnant vixens of the placebo and not pregnant vixens of the BCD group are not shown in Fig. 5 because from day 2 before first AI to day 0, respectively, these vixens were under the influence of treatment.

In Fig. 6, a bar chart shows the stage of heat at the initiation of treatment, the number of days from initiation of treatment to AI and the outcome (pregnant or not pregnant) in 70 inseminated vixens. In this graph, all vixens showing sonographic signs of

**Table 1**

Inseminated (n), pregnant (n, %) and whelping (n/%) vixens in group A (placebo), and finrozole treated groups B (0.5 mg/kg), C (3.5 mg/kg), and D (24.5 mg/kg).

Group	Inseminated	Pregnant		Whelping	
	n	n	%	N	%
A	19	17	89.5	16	84.2
B	16	13	81.3	8	50.0
C	18	10	55.6	9	50.0
D	17	9	52.9	6	35.3
Total	70	49	70.0	39	55.7



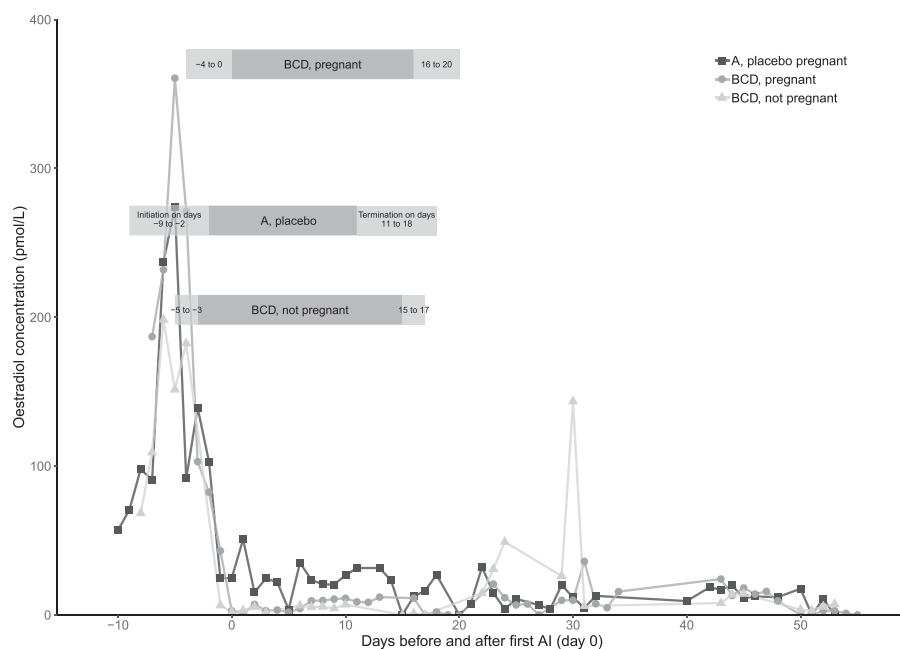
**Fig. 2.** Vaginal electrical resistance ( $\Omega$ ) before first AI in vixens that became pregnant (A, placebo  $n = 17$  and BCD  $n = 32$ ) and in treated vixens that did not become pregnant (group BCD  $n = 19$ ). Values are depicted as means  $\pm$  SD. Horizontal bars indicate the treatment periods of individual vixens.

pregnancy were considered to be pregnant irrespective of whether or not pups were delivered.

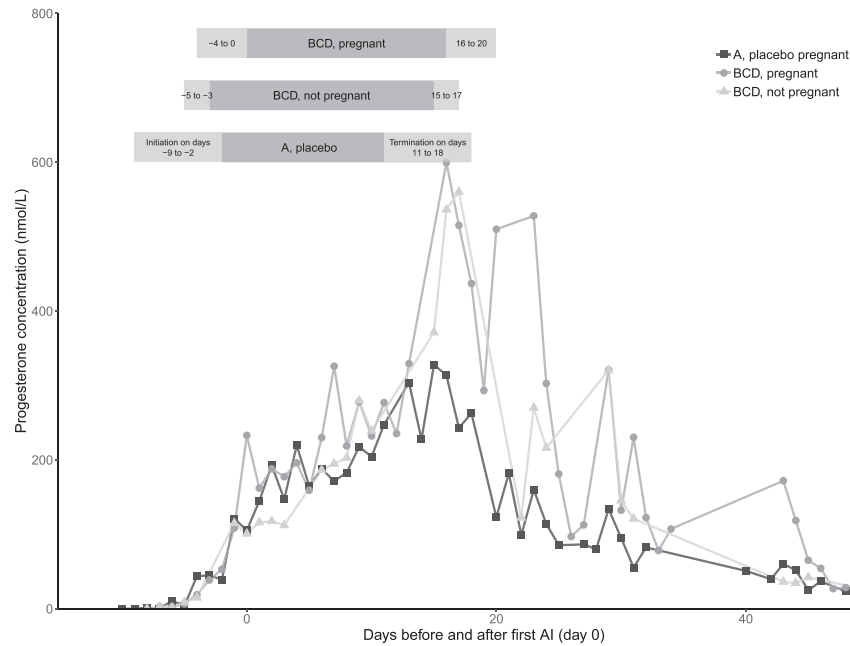
Pregnancy rates were about equal in group A (placebo 89.5%) and group B (81.3%), but markedly reduced in treatment groups C (55.6%) and D (52.9%) (Tables 1 and 2). For vixens that were inseminated only once, the pregnancy rates in different groups were 85.7% (A), 72.7% (B), 46.7% (C) and 41.7% (D). The results of this study clearly indicate that administration of finrozole significantly prevented pregnancy if the treatment with finrozole at a dosage of 3.5 mg/kg or 24.5 mg/kg was initiated at least 4 days

before first AI, corresponding with 2–5.5 days before estimated ovulation [14].

Whelping rates (percentages of inseminated vixens that whelped) in different groups were 84.2% (16/19;  $n$  whelping/ $n$  inseminated; A), 50.0% (8/16; B), 50.0% (9/18; C) and 35.3% (6/17; D). In the entire BCD group, 45.1% of the vixens whelped (23 whelping/51 inseminated). Numbers of pups live at birth in different groups were 151 (A), 55 (B), 52 (C) and 23 (D). The mean numbers of pups live at birth per inseminated and whelping vixens are presented in Table 3. Litter size ranged in different



**Fig. 3.** Mean oestradiol concentrations (pmol/L) throughout the experimental period in vixens that became pregnant (A, placebo  $n = 17$  and BCD  $n = 32$ ) and in treated vixens that did not become pregnant (group BCD  $n = 19$ ). Horizontal bars represent the 21 days treatment period and indicate the treatment periods of individual vixens.



**Fig. 4.** Mean progesterone concentrations (nmol/L) throughout the experimental period in vixens that became pregnant (A, placebo  $n = 17$  and BCD  $n = 32$ ) and in treated vixens that did not become pregnant (group BCD  $n = 19$ ). Horizontal bars represent the 21 days treatment period and indicate the treatment periods of individual vixens.

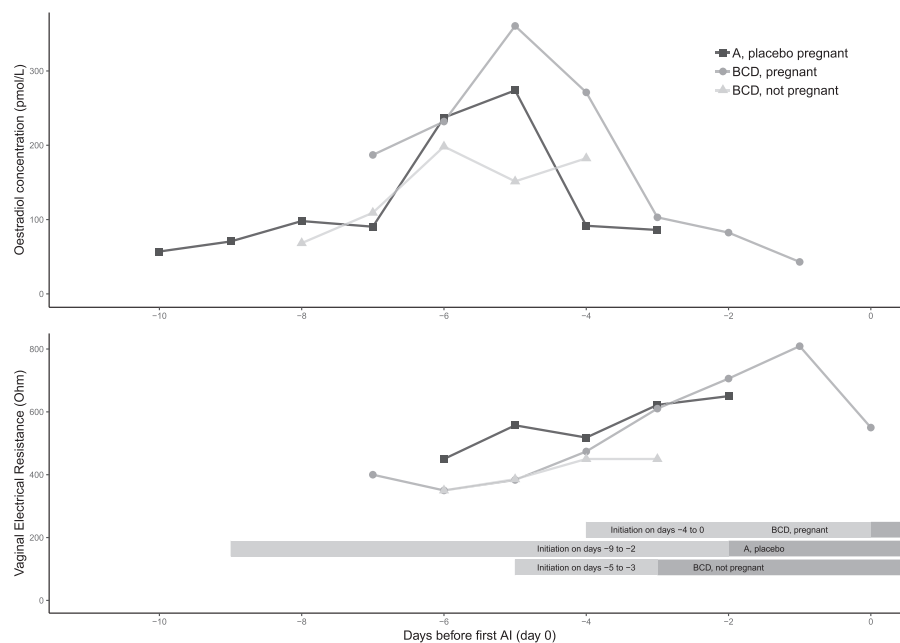
groups from 4 to 13 (A), 4 to 12 (B), 2 to 10 (C) and 1 to 9 (D) pups live at birth.

The percentages of whelping vixens experiencing pup loss between birth and one week thereafter was 68.8% in group A, 37.5% in group B, 55.6% in group C, and 33.3% in group D. There was no difference in pup loss between placebo and treatment groups ( $P = 0.111$ ). In litters of 19 vixens (11 A, 3 B, 4 C and 1 D group vixens) pups were lost in the first week of life. In one vixen of both group C

and group D the entire litter was affected. The percentages of pups lost were 23.8% in group A, 5.5% in group B, 11.5% in group C, and 8.7% in group D.

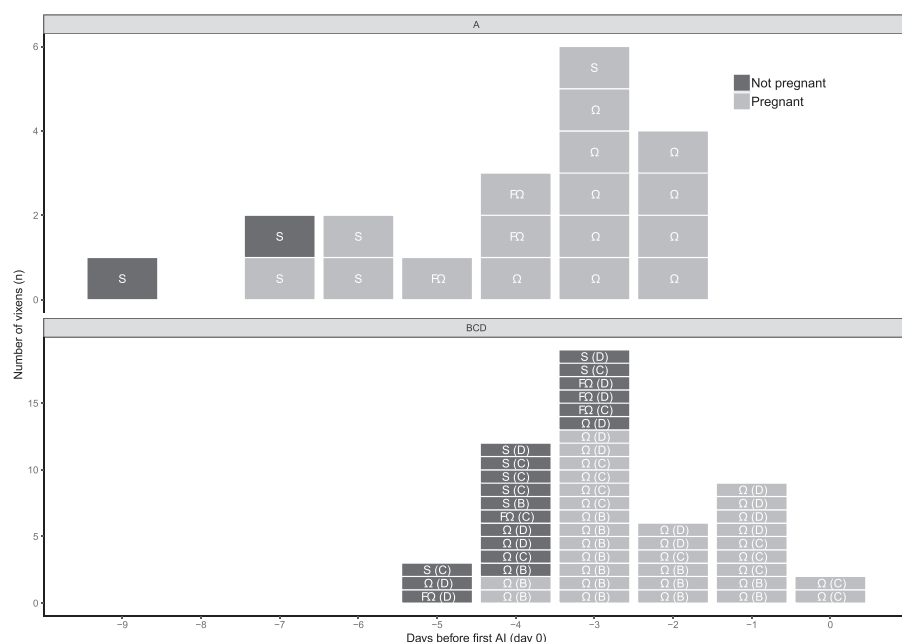
#### 4. Discussion

We report a promising protocol for using aromatase inhibition to prevent pregnancy in the farmed blue fox. The success is based



**Fig. 5.** Mean oestradiol concentrations (pmol/L) in relation to vaginal electrical resistance ( $\Omega$ ) before first AI in vixens that became pregnant (A, placebo  $n = 17$  and BCD  $n = 32$ ) and in treated vixens that did not become pregnant (group BCD  $n = 19$ ). Oestradiol concentrations and VER values originate from measurements performed before the initiation of treatment corresponding to the first two blood collections and VER measurements of vixens registered before the initiation of treatment. Horizontal bars indicate the treatment periods of individual vixens.





**Fig. 6.** Illustration of the number of treatment days before first AI in individual vixens of group A (placebo  $n = 19$ ) and group BCD (finrozole  $n = 51$ ) together with the outcome of insemination (pregnant: group A  $n = 17$ , group BCD  $n = 32$  vs. not pregnant: group A  $n = 2$ , group BCD  $n = 19$ ). Symbols in boxes indicate the stage of heat at the initiation of treatment. S = swelling of the vulva (all three scales), FΩ = first day of ohm measurement, Ω =  $\geq 2$  days of ohm measurements. (B), (C), (D) indicate group affiliation of vixens.

on the two highest doses of treatment (3.5 mg/kg and 24.5 mg/kg) that prevented pregnancy in the experimental vixens if treatment was initiated at least four days before first AI (Tables 1 and 2). To the authors' knowledge, this is the first time a farmed fox was used as a model for a domestic dog in a drug dose determination study to prevent pregnancy. As Table 2 shows, there was considerable variation in the treatment response in terms of the pregnancy outcome, depending on the timing of onset of the treatment. If the treatment was initiated early enough and if the dose was high enough, the vixen did not become pregnant. The time limit for this was found to be four days or earlier in relation to the expected AI. This can be assessed to be up to 5.5 days before estimated ovulation [14]. However, if the treatment was initiated one day later, the response was variable with only 33–50% of the vixens responding and therefore not becoming pregnant (Table 2). If the treatment was initiated later than this, aromatase inhibition did not work and the treated vixens became pregnant.

There were two probable reasons for the earlier than expected onset of cyclicity for the experimental vixens that somewhat complicated our experimental setting. Firstly, handling and moving vixens about within the farm, which was initiated already in February, is known to hasten the onset of the breeding season [16]. Secondly, there are no efficient oestrus synchronization protocols available for the fox. Therefore, due to this handling effect in the females, the onset of natural heat occurred earlier than predicted and we had more vixens than intended with initiation of treatment in progressed pro-oestrus or even in oestrus. Moreover, it is also possible that at initiation of treatment, in some vixens, ovulation might have occurred and oocytes were under maturation and even ready for fertilization. In this study, the interval from initiation of treatment to first AI was on average between three to four days which is clearly shorter than the average duration of pro-oestrus in the vixens [14].

Regarding the VER values, characteristic profiles for blue fox vixens were observed in this study. No statistically relevant differences were established between the treatment groups. It is well

known, that individual differences exist regarding the duration of the period of high VER values (range one to three days [18]). Due to this, as AIs were performed during the period of high VER, there was a discrepancy in the number of AIs per vixen resulting in one or two AIs in all except one vixen which had four AIs due to a two-phase resistance curve. This routine AI protocol was accepted in this study to provide all inseminated vixens an equal opportunity to become pregnant. The vixens with two or more AIs were equally distributed within each treatment group (3–5 per each) so their effect is similar in all groups. In fox farming, VER values are the only indicator used for prediction of ovulation in vixens. As described previously by Møller et al. [18] and Farstad et al. [17], the VER usually reaches maximum values around ovulation i.e., approximately two to three days after the oestradiol peak or half a day after the LH peak [18]. A subsequent decrease of at least 50 Ω is considered significant [14].

In the present study, no statistically significant differences were observed in plasma oestradiol concentrations between placebo and the treatment groups. Plasma levels of oestradiol-17-β are known to increase from approximately one week prior to the LH peak. Maximum oestradiol levels of 40–120 pg/mL (140–350 pmol/L) are detected one day prior or on the day of the LH peak [17,18]. In this study, increasing levels of oestradiol were measured starting day -10 before the first AI, reaching peak values up to 400 pmol/L in the placebo and BCD pregnant groups, five days prior to the first AI. In the BCD not pregnant group, a lower peak of approximately 200 pmol/L was observed 4 days before first insemination. After peak values, coinciding with the time of initiation of treatment, oestradiol levels decreased to below 50 pmol/L approximately one day before the first AI, maintaining low values during the luteal phase. Oestradiol levels have been found to decrease rapidly following the LH peak in vixens and remain low for several weeks during the luteal phase [14]. Our results are similar and therefore, in BCD pregnant vixens, the decrease in oestradiol concentration during the period day -5 to the first AI may have occurred due to the physiological decrease after the oestradiol and/or LH peak

**Table 2**

Pregnancy rates (%) in the individual treatment groups (A, B, C, D) and in the total of treated vixens (BCD) according to the number of days between initiation of treatment and first AI.

Days between initiation of treatment and first AI	Pregnancy rate (n pregnant/n total inseminated) in group				
	A n = 19	B n = 16	C N = 18	D n = 17	BCD n = 51
0			100.0 (2/2)		100.0 (2/2)
1		100.0 (2/2)	100.0 (3/3)	100.0 (4/4)	100.0 (9/9)
2	100.0 (4/4)	100.0 (3/3)	100.0 (1/1)	100.0 (2/2)	100.0 (6/6)
3	100.0 (6/6)	85.7 (6/7)	66.7 (4/6)	50.0 (3/6)	68.4 (13/19)
4	100.0 (3/3)	50.0 (2/4)	0.0 (0/5)	0.0 (0/3)	16.7 (2/12)
5	100.0 (1/1)		0.0 (0/1)	0.0 (0/2)	0.0 (0/3)
6,7,9	60.0 (3/5)				
Total	89.5 (17/19)	81.3 (13/16)	55.6 (10/18)	52.9 (9/17)	62.7 (32/51)

AI = artificial insemination. Group A (placebo), group B (0.5 mg/kg), group C (3.5 mg/kg) and group D (24.5 mg/kg).

rather than due to initiation of treatment with finrozole. Instead, in the BCD not pregnant vixens, oestradiol concentration had not reached a physiological peak at the initiation of treatment (in fact, the values were still increasing as shown in Fig. 5) and therefore, in this group, finrozole treatment may have influenced the oestradiol profile and caused a decrease in oestradiol concentrations earlier than it would physiologically have occurred.

Extraordinary high concentrations of oestradiol were found in the 9th blood sample in two vixens of group C (667.2 and 644.9 pmol/L). On the other hand, three vixens (two in group C and one in group D) had minor increases from 100 to 165 pmol/L in the 9th blood sample. In the latter group, one group C vixen had two pups, the other group C vixen was negative in ultrasound and had no pups and the group D vixen had two fetuses in ultrasound but did not whelp. As the oestradiol increase was observed in both, not pregnant and pregnant vixens, a causal connection with a recurrence of heat seems doubtful. This is supported by the fact, that in all vixens the progesterone patterns indicated a normal and continuous luteal phase.

In this study, no statistically significant differences were found in peripheral progesterone values between treatment and placebo groups. In each group, a rise in plasma progesterone values was observed prior to first AI. The mean time period of treatment before the first AI was  $3.1 \pm 1.6$  days. In the placebo and BCD pregnant groups, the first increase in progesterone concentration together with the initial decrease of the oestradiol concentration was observed five days before the first AI. This is in agreement with Farstad [14] who stated that after a rapid decrease in oestradiol levels progesterone levels increase rapidly from the first initial rise simultaneously with the LH peak. The average progesterone values at the time of insemination ranged between 100 and 230 nmol/L. According to Urlings et al. [28] and Høier [29], progesterone values from 95 to 143 nmol/L indicate that first AI should occur one day after blood sampling and values above 143 nmol/L indicate that first AI should be performed on the day of blood sampling. Based on this, the timing of AIs in this study provided good prerequisites for fertilization.

In all groups, peak progesterone values ranging from 300 to 600 nmol/L were measured around 16–17 days after first AI.

Thereafter progesterone values began to decrease. In earlier studies [30–32], the peak progesterone values were lower, reaching a plateau five to 12 days after mating and remained high for approximately two weeks. A clear plateau was not observed in the present study. In addition, compared to the placebo group, a second peak in progesterone concentrations seemed to occur in both treatment groups approximately 28 days after the first AI. No such peak was observed in the placebo group. A similar progesterone pattern has been observed in unpublished studies on finrozole treatment in female dogs. The second progesterone peak still needs clarification.

From the results of the present study, it can be hypothesized that prevention of oestradiol production by finrozole may have caused a reorientation of steroid metabolism to other paths and therefore, resulted in high progesterone values. In cattle, after treatment with the non-steroidal aromatase inhibitor letrozole, higher than normal progesterone values were detected [10–12]. In addition, administration of aromatase inhibitors may alter the ovulatory process. In rats, administration of letrozole resulted in polycystic ovaries and caused incomplete luteinization and a decreased number of corpora lutea [33]. In our study, the ovaries of the vixens were not examined. However, as already mentioned above, in all vixens the progesterone profiles appeared normal, indicating the presence of either corpora lutea or luteinized follicles (reported earlier by Farstad et al. [17]).

Regarding the assessment of hormone patterns, an influence of stress caused by handling of the vixens must be taken into consideration. In a study on silver foxes that was started in animals not used to handling, manipulations resulted in long-term stress, high levels of fear-responses and enlarged adrenals [34]. Being taken out of their cage with a neck-tongue should be regarded as a stressor to vixens [35]. In our study, all vixens were primi- or multiparous and therefore, experienced to handling prior to the beginning of the study and familiar with the farm environment and personnel.

The blood sampling was executed in anaesthetized vixens. In a study in mice, the effects of both single and repeated anaesthesia (six times at an interval of three to four days) were considered mild

**Table 3**

Numbers of live pups (mean  $\pm$  SD) counted at birth in the placebo and the individual treatment groups in relation to inseminated and whelping vixens.

Number of live pups		
Group	/Inseminated vixen	/Whelping vixen
A	7.9 $\pm$ 4.8, n = 19	9.4 $\pm$ 3.6, n = 16
B	3.5 $\pm$ 4.5, n = 16	6.9 $\pm$ 4.1, n = 8
C	2.9 $\pm$ 3.6, n = 18	5.8 $\pm$ 3.0, n = 9
D	1.4 $\pm$ 2.4, n = 17	3.8 $\pm$ 2.8, n = 6

Group A (placebo), group B (0.5 mg/kg), group C (3.5 mg/kg) and group D (24.5 mg/kg).

[36]. Nevertheless, the duration of increased stress and anxiety levels were assessed to be longer in mice that were repeatedly anaesthetized. Short-term acute stressors often fail to affect reproduction [37]. However, it seems possible that several times repeated short-term handling stress during health inspections and blood collections before and during pregnancy may have influenced the reproductive outcome and increased perinatal pup loss as was described for handling stress [38].

In the present study, 10 vixens diagnosed pregnant in ultrasound were not observed giving birth. During pregnancy, blue fox vixens are known to experience both embryonic and foetal losses [39]. They may also demolish born cubs, although infanticide is not a common problem in blue foxes [27]. Naturally occurring post-implantation pregnancy losses of 27.5% are reported for blue foxes [39]. In our study, although the vixens were handled several times during pregnancy, the proportion of lost pregnancies (20.4%) was lower. The lost pregnancies were probably due to vixens experiencing resorption or giving birth to weak pups that died and were eaten by their mothers. Abortions cannot be completely excluded. However, abortion seems to occur seldom in blue fox vixens (0.5% of barren vixens), thus indicating that resorption of embryos may be a major factor affecting barrenness [40].

In the placebo group, the average number of live pups per whelping vixen was 9.4. This is in agreement with other authors reporting eight to ten live and dead pups per whelping vixen on the first day postpartum [41–43]. In the treatment groups BCD, the average number of live pups per whelping vixen varied from 3.8 to 6.9. The total number of live born pups in the three treatment groups was 130 compared to 151 in the placebo group. Furthermore, in the treatment groups the litter size tended to be smaller the longer the interval between initiation and first AI. If treatment started less than 3 days before first AI, vixens had a maximum of 9–12 pups, if the interval between start of treatment and first AI was 3 days or more, the maximum litter size was 2–6 pups.

Partial ( $n = 19$ ) or complete ( $n = 2$ ) losses of neonates occurred in 53.8% of 39 litters within the first week of life. No deleterious effects (for instance malformations) of finrozole on pups could be verified. Interestingly, postnatal pup loss was more frequent in the placebo group (23.8%) than in the treatment groups (5.5–11.5%). The amount of losses was in accordance with the result of Pyykönen et al. [44] who reported 32.7% and 16.7% of pup losses in primiparous and multiparous vixens, respectively, from birth to weaning.

In the present study, the efficacy of the treatment was significantly affected by the stage of heat at the initiation of drug administration (Fig. 6). Vixens that were inseminated three, four or five days after the start of treatment with 3.5 mg/kg or 24.5 mg/kg in an early stage of follicular development did not conceive. If administration of finrozole was initiated close to AI (two to zero days before first AI), none of the doses was successful for achieving contraception as the stage of heat was too advanced.

## 5. Conclusions

According to the results of this study, pregnancy in blue fox vixens can be prevented by finrozole as long as treatment at doses of 3.5 mg/kg or 24.5 mg/kg is initiated at least four days before the first AI. Vixens that have not reached the oestradiol peak are susceptible to the treatment. Thus, careful detection of initial clinical signs of heat will be required, which in bitches can usually be achieved with little or no stress. Therefore, based on the results shown in this study, the authors conclude that the use of aromatase inhibitors could prove a promising tool for contraception in female dogs in the future.

## CRedit authorship contribution statement

**L. Lindh:** Writing - original draft, Writing - review & editing, Visualization, Formal analysis, Data curation. **H. Lindeberg:** Methodology, Investigation, Writing - original draft, Writing - review & editing, Formal analysis, Data curation. **A. Banting:** Conceptualization, Methodology, Writing - review & editing. **S. Banting:** Conceptualization, Project administration. **S. Sainmaa:** Investigation, Methodology, Writing - review & editing. **S. Beasley:** Funding acquisition, Investigation, Writing - review & editing. **H.T. Korhonen:** Resources, Investigation, Supervision, Writing - review & editing. **O.A.T. Peltoniemi:** Writing - original draft, Writing - review & editing, Formal analysis, Data curation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2020.04.009>.

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